FEBS Letters 354 (1994) 187–190

FEBS 14736

Selectivity of DNA polymerases toward α and β nucleotide substrates of D and L series

Dmitry G. Semizarov[a,*], Lyubov S. Victorova[a], Natalia B. Dyatkina[a], Martin von Janta-Lipinski[b], Alexander A. Krayevsky[a]

[a]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Str., Moscow 117984, Russian Federation*
[b]*Max-Delbrück Centre of Molecular Medicine, Berlin D-13125, Germany*

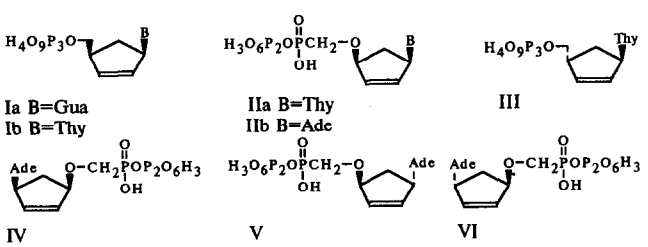
Received 20 September 1994

Abstract The substrate properties of four carbocyclic D and L nucleoside 5′-triphosphate analogs toward HIV and AMV reverse transcriptases and terminal deoxynucleotidyl transferase were evaluated. The compounds of the D-β and L-β series were found to be terminating substrates for these enzymes, while the derivatives of the D-α and L-α series were recognized only by terminal deoxynucleotidyl transferase, suggesting that for the template-independent enzyme the mutual orientation of the two fragments is of no significance. A hypothesis for binding of nucleotides to the DNA polymerase active center was proposed.

Key words: Reverse transcriptase; Terminal deoxynucleotidyl transferase; Modified nucleoside 5′-triphosphate; Stereomer

1. Introduction

The role of the sugar residue in the interaction between dNTP and the (template–primer–DNA polymerase) complex has been extensively studied. In recent years there has been substantial accumulation of data on the effect of substituents in the sugar residue, as well as its conformation and, finally, the configuration of the nucleoside on the process of binding to DNA polymerase [1,2]. 2′,3′-Dideoxy-2′,3′-didehydronucleoside 5′-triphosphates have been shown to be substrates for retroviral RTs and some other DNA polymerases [3,4]. The carboanalogs of these compounds (I,II) displayed similar substrate properties [5–7].



As a rule, dNTP analogs containing a double C2′-C3′ bond exhibited high affinity to the DNA synthesizing complex regardless of what template-primers and DNA polymerases were used. Van Draanen et al. have studied the substrate properties of the stereomers IIa and III toward HIV RT and shown that the L isomer has a 50-fold lower affinity to the enzyme [7].

We evaluated four carbocyclic dATP analogs containing a double C2′-C3′ bond (IIb, IV–VI) as substrates for HIV and AMV RTs and TdT using DNA and RNA templates.

2. Materials and methods

Triphosphates IIb and IV–VI were synthesized from the corresponding nucleosides. Synthesis of the nucleosides has been described in [8], HIV RT was isolated according to [9], AMV RT and calf thymus TdT were from Omutninsk Chemicals and Amersham, respectively. Phage M13mp10 DNA was isolated according to [10]. Heteropolymeric RNA was synthesized by run-off transcription of *Sal*GI-digested plasmid pPV-19 with T7 RNA polymerase according to [11]. The plasmid containing a fragment of pBR322 DNA between the *Sal*G1 and *Sph*I sites was a gift of Dr. S. Kochetkov. Primer extension and sequencing assays were performed as described in [12]. For kinetic assays, the reaction mixture (6 μl) contained 0.02 μM DNA- primer (Fig. 1a) or 0.2 μM RNA-primer (Fig. 1b,c) complex, 3 units of AMV or HIV RT or 2 units of TdT, dNTP or its analog at different concentrations, and the appropriate buffer [12]. The reaction was carried out for 2 min at 370°C and terminated by adding 3 μl of formamide with EDTA. The products were separated by PAGE, and the autoradiographs were scanned in an LKB densitometer.

3. Results

The analogs were evaluated as terminating substrates on DNA (Fig. 1a) and RNA (Fig. 1c) templates (Fig. 1). Fig. 2 presents the results of the primer extension assay for dATP, IIb, and IV–VI. It can be seen that IIb and IV were efficiently incorporated into the DNA chain by HIV RT, whereas VI displayed weaker substrate properties and V was not recognized by the enzyme. A similar picture was observed with the RNA template. The results of AMV RT assays were similar but V and VI were not incorporated into the DNA chain at all (data not shown).

We also evaluated the ability of IIb and IV to terminate DNA synthesis catalyzed by HIV RT on DNA (Fig. 1d) and RNA (Fig. 1b) templates. Fig. 3 presents the termination pattern for IIb obtained with complex d. In control assays (lanes 3–10) d_2NTPs were used as chain terminators. Clearly, DNA synthe-

*Corresponding author.

Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; AMV, avian myeloblastosis virus; TdT, terminal deoxynucleotidyl transferase; dNTP, 2′-deoxynucleoside 5′-triphosphate; d2NTP, 2′,3′-dideoxynucleoside 5′-triphosphate.

SSDI 0014-5793(94)01123-0

```
                10        20        30
a)    d(GGGTCAGTGCTGCAACATTTTGCTGCCGGT...)
       [5'-32P]d(GACGTTGTAAAACG)

                10        20
b)   r(CGAGAGGGAAUACGCUGAGGACGUAA...)
       [5'-32P]d(ATGCGACTCCTGC)

                    10        20
c)        r(CGAGAGGGAAUACGCUGAGGACGUAA...)
  [5'-32P]d(GCTCTCCCTTATGCGACTCC)

                  10        20        30        40
d)      d(GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA...)
[5'-32P]d(CCCAGTCACGACGT)
```

Fig. 1. Template–primer complexes used in this work.

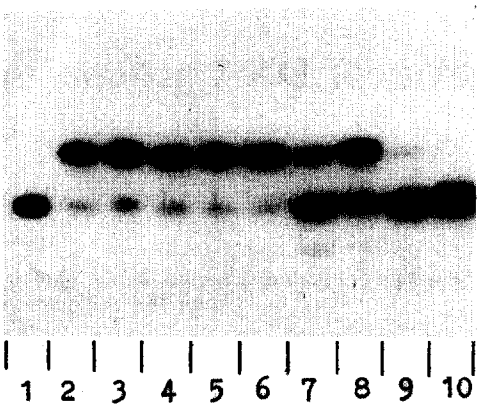


Fig. 2. Primer extension catalyzed by HIV RT. 1, Template–primer (complex a) + enzyme; 2, as 1 + 10 μM dATP; 3, as 1 + 10 μM IIb; 4, as 1 + 100 μM IIb; 5, as 1 + 10 μM IV; 6, as 1 + 100 μM IV; 7, as 1 + 10 μM VI; 8, as 1 + 100 μM VI; 9, as 1 + 10 μM V; 10, as 1 + 100 μM V.

sis is terminated at the IIb/dATP concentration ratio of 2–12. Results were similar for IV, both compounds produced similar pictures on DNA and RNA templates (data not shown).

To assess the incorporation quantitatively, we obtained the time and concentration dependences of product formation using DNA and RNA templates. The time dependence was linear up to 4.5 min. The concentration dependence was obtained at 2 min and used to construct the Lineweaver–Burk plot and determine the K_m values and V_{max}/V_{max}(dATP). In some control assays (complex c), d_2ATP was used instead of dATP because otherwise the assay mixture would contain all four dNTPs and the nascent chain would be further elongated. The K_m values for V and VI in RNA-dependent primer extension catalyzed by AMV RT ranged from 1.5 to 3.0 μM.

It can be seen in Fig. 4 that all four tested compounds are recognized by TdT and extend the primer chain, IIb and IV being better substrates than V and, especially, VI. Kinetic assays with TdT were carried out within the linear portion of the kinetic curve, the reaction time was 2 min. The kinetic constants for all four compounds in TdT-catalyzed primer extension are summarized in Table 2.

4. Discussion

Using DNA and RNA templates, we showed that IIb and IV are good terminating substrates for AMV and HIV RTs. Their K_m values are close to each other and rather small, implying high affinity of the compounds to RTs. It is noteworthy that our data are inconsistent with some results obtained in other laboratories. In particular, it has been found that the D isomer Ib displays a 50-fold higher affinity to HIV RT than the L isomer III [7]. It has been also shown that the L isomer of 2′-deoxy-3′-thio-5-fluorocytosine 5′-triphosphate is a better substrate for HIV RT [13] and human hepatitis B virus DNA polymerase [14] than the D isomer.

The results obtained in this work are in agreement with our data on modified dNTPs with conformationally rigid flattened glycones [3,5,7] (for more references see [6]) and support the hypothesis that such compounds fit the structure of the RT active center modelling the transition state of dNTPs in the RT–dNTP complex [15]. The results presented here, as well as our earlier data [3,5,7,15,18–20] indicate that the absence of the 3′ hydroxyl does not inactivate the nucleotide substrate.

It seems likely that V and VI are not recognized by RTs. The primer extension observed in the presence of these compounds is presumably due to the admixture of IIb and IV because the difference in the retention times for the IIb-V and IV-VI pairs upon HPLC chromatography is about 1 min [8] and their K_m values differ 50–200-fold.

At the same time, all four examined compounds were incorporated into the primer chain by TdT and their K_m values in TdT-catalyzed primer extension differed only slightly.

Thus, RTs recognize only '*cis*-like' isomers (IIb and IV), while template-independent TdT incorporates both '*cis*-like' (IIb and IV) and '*trans*-like' isomers (V and VI) into the primer chain with a similar efficiency. These data suggest that for the template-independent enzyme, tight fixation of the nucleic base with respect to the triphoshate residue is not required. Indeed, binding of dNTP to the (TdT–primer) complex does not include Watson–Crick pairing.

Based on the analysis of our results and the data published in the literature, we suggest that the glycone of dNTP functions

Table 1
Kinetic constants for IIb and IV in primer extension catalyzed by AMV and HIV RTs

	Enzyme + template-primer							
	AMV RT + complex a		AMV RT + complex b		AMV RT + complex c		HIV RT + complex c	
	K_m, μM	V_{max} / V_{max}(dATP)	K_m, μM	V_{max} / V_{max}(dATP)	K_m, μM	V_{max} / V_{max}(d_2ATP)	K_M, μM	V_{max} / V_{max}(d_2ATP)
IIb	0.03 ± 0.004	0.72	0.028 ± 0.003	0.63	0.069 ± 0.05	1.38	0.021 ± 0.002	1.26
IV	0.019 ± 0.002	0.77	0.0076 ± 0.0004	0.77	0.014 ± 0.001	1.51	0.0034 ± 0.001	1.52
ddATP	–	–	0.21 ± 0.05	0.83	0.19 ± 0.03	1.00	0.19 ± 0.04	1.00
dATP	1.72 ± 0.14	1.00	0.034 ± 0.003	1.00	–	–	–	–

Table 2
Kinetic constants for IIb and IV-VI in primer extension catalyzed by TdT

	IIb	IV	V	VI
K_m, μM	3.1 ± 0.3	5.0 ± 0.2	10.1 ± 0.4	5.2 ± 0.2
V_{max}/V_{max} (IIb)	1.00	0.88	0.45	0.76

only as a framework positioning the nucleic base and the triphosphate residue correctly relative to each other and does not bind to the active center, whereas the latter are directly involved in specific binding. The glycone maintains the correct distance and torsion angle between the glycoside and C4′–C5′ bonds, thus ensuring efficient binding to RTs. Flattening of the glycone structure facilitates rotation of the nucleic base with respect to the glycone, providing thermody-namically more efficient Watson–Crick pairing [21,22].

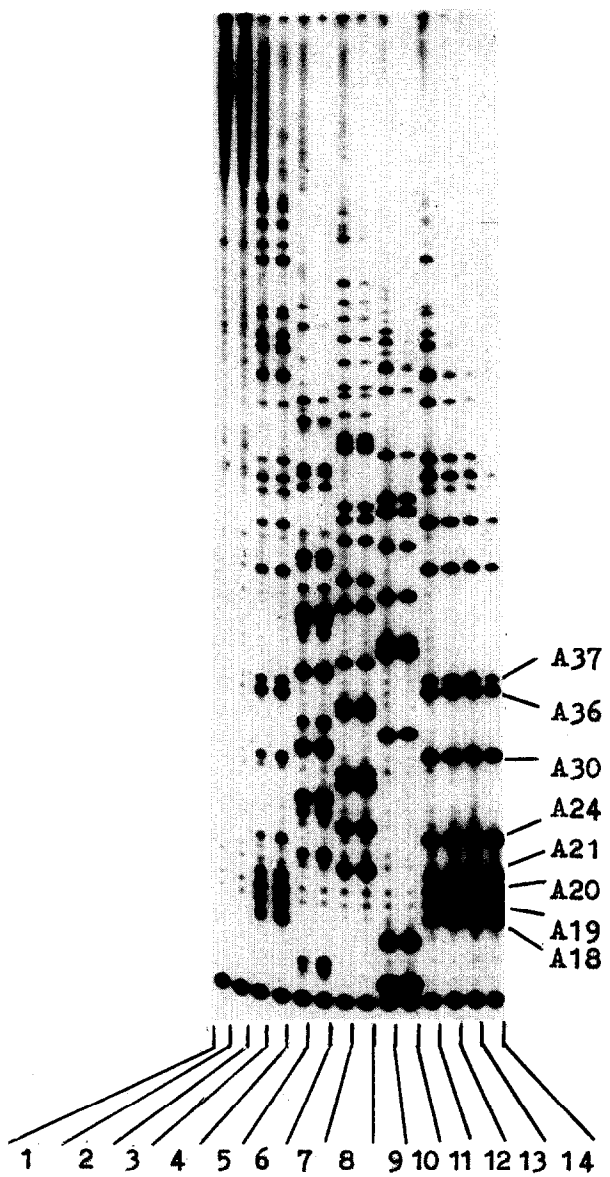


Fig. 3. Termination of DNA synthesis catalyzed by HIV RT. 1,2, DNA synthesis in the absence of terminators; 3–10, in the presence of 1 μM (3) and 2 μM (4) d_2ATP; 1 μM (5) and 2 μM (6) d_2GTP; 1 μM (7) and 2 μM (8) d_2CTP; 2 μM (9) and 4 μM (10) d_2TTP; 11–14, in the presence of 5 μM (11), 15 μM (12), 20 μM (13), and 30 μM (14) IIb.

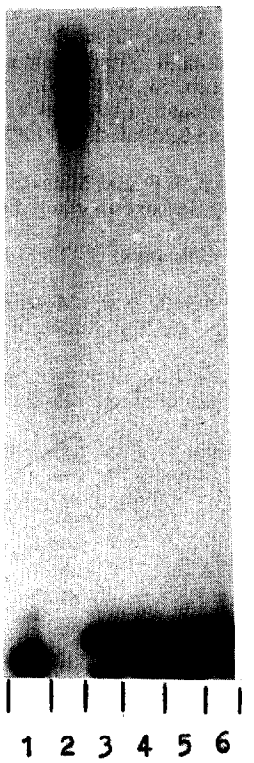


Fig. 4. Primer extension catalyzed by TdT. 1, primer + enzyme; 2, as 1 + 10 μM dATP; 3, as 1 + 10 μM IIb; 4, as 1 + 10 μM IV; 5, as 1 + 10 μM VI; 6, as 1 + 10 μM V.

Compounds IIb and IV may be useful in designing antiviral agents because they, first, display high affinity to HIV RT and, second, contain enzymatically nonhydrolyzable bonds between the nucleic base, glycone, and triphosphate residue. We are currently working on modification of the β and γ phosphates in IIb and IV that would additionally increase their stability in blood.

It has been shown that the level of TdT in leukocytes of leukemia patients is very high [23]. Compounds V and VI proved to be specific terminating substrates for TdT. Therefore, we are planning to evaluate the corresponding monophosphonates as inhibitors of TdT in cell cultures.

Acknowledgments: This work was supported by the Russian State Fund for Basic Research (Grant 93-04-20542).

References

[1] Krayevsky, A.A., Kukhanova, M.K., Atrazhev, A.M., Chidgeavadze, Z.G. and Beabealashvilli, R.Sh. (1987) Mol. Biol. 21, 33–38.
[2] Wright, G.E. and Brown, N.C. (1990) Pharmacol. Ther. 47, 447–497.
[3] Dyatkina, N., Minassyan, Sh., Kukhanova, M.K., Krayevsky, A., von Janta Lipinski, M., Chidgeavadze, Z. and Beabealashvilli, R. (1987) FEBS Lett. 219, 151–155.
[4] Matthes, E., Lehmann, Ch., Scholz, D., von Janta Lipinski, M., Gaertner, K., Rosenthal, H. and Langen, P. (1987) Biochem. Biophys. Res. Commun. 148, 78–85.
[5] White, E.L., Parker, W.B., Macy, L.J., Shaddix, S.C., McCaleb, G., Secrist III, J.A., Vince, R. and Shannon, W.M. (1989) Biochem. Biophys. Res. Commun. 161, 393–398.
[6] Coe, D.M., Roberts, S.M. and Storer, R. (1992) J. Chem. Soc. Perkin Transact. 2695–2704.

[7] Van Draanen, N.A., Tucker, S.C., Boyd, F.L., Trotter, B.W. and Reardon, J.E. (1992) J. Biol. Chem. 267, 2509–25024.
[8] Dyatkina, N., Costisella, B., Theil, F. and von Janta-Lipinski, M. (1994) Tetrahedron Lett. 35, 1961–1964.
[9] Rosovskaya, T.A., Belogurov, A.A., Lukin, M.A., Chernov, D.N., Kukhanova, M.K. and Beabealashvilli (1993) Mol. Biol. (Russian) 27, 618–630.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Sampson, J.R. and Uhlenbeck, O. (1988) Proc. Natl. Acad. Sci. USA 85, 1033–1036.
[12] Victorova, L.S., Dyatkina, N.B., Mozzherin, D.Ju., Atrazhev, A.M., Krayevsky A.A. and Kukhanova, M.K. (1992) Nucl. Acids Res. 20, 783–789.
[13] Wilson, J.E., Martin, J.L., Borrota-Esoda, K., Daurs, M.G., Hopkins, S.E., Painter, G., Liotta, D. and Furman, P.A. (1993) Antiviral Res. 20, Suppl. 1, 75.
[14] Chang, C.-N., Skalski, V., Jhou, J.H. and Cheng, Y.-C. (1992) J. Biol. Chem. 267, 22414–22420.
[15] Semizarov, D.G., Victorova, L.S., Krayevsky, A.A., Kukhanova, M.K. (1993) FEBS Lett. 327, 45–48.
[16] Krayevsky, A.A., Kukhanova, M.K., Atrazheva, E.D., Dyatkina, N.B., Papchikhin, A.V., Chidgeavadze, Z.G. and Beabealashvilli, R.Sh. (1988) Nucleosides Nucleotides 7, 613–617.
[17] Reardon, J.E. (1992) Biochemistry 11, 4473–4479.
[18] Krayevsky, A.A. and Watanabe, K.A. (1993) Nucleosides Nucleotides 12, 649–670.
[19] Krayevsky, A.A., Victorova, L.S., Mozzherin, D.Yu. and Kukhanova, M.K. (1993) Nucleosides Nucleotides 12, 83–93.
[20] Shirokova, E.A., Tarussova, N.B., Shipitsin, A.V., Semizarov, D.G. and Krayevsky, A.A., J. Med. Chem. in press.
[21] Gurskaya, G.V., Bochkarev, A.V., Zhdanov, A.S., Dyatkina, N.B. and Krayevsky, A.A. (1991) Intern. J. Pur. Pyr. Res. 2, 55–60.
[22] Van Roey, P., Taylor, E.W., Chu, C.K. and Schinazi, R.F. (1993) J. Am. Chem. Soc. 115, 5365–5371.
[23] Spigelman, Z., Duff, R., Beardsley, G.P., Broder, S., Cooney, D., Lavden, M.R., Mitsuya, H., Ullman, B. and Mc Caffrey, R. (1988) Blood 71, 1601–1608.